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Evaluation of 'CAM-1,' a Warning Device for Organophosphate Hazardous Material Spills

Midwest Research Inst, Kansas City, Mo

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16. ABSTRACT

The Cholinesterase Antagonist Monitor, CAM-1, which uses immobilized cholinesterase on a porous polyurethane pad in an electrochemical cell is able to sense the presence of low concentrations of cholinesterase inhibitors in water on a real time basis. The sensitivity of CAM-1 to organophosphates and carbamates is a function of the affinity of cholinesterase for them; thus its response is greater for those compounds which are more toxic (i.e., the detectable concentrations are inversely proportional to the LD_{50}s of these pesticides). Studies were conducted to show the sensitivity of the monitor to 22 organophosphates and 7 carbamate pesticides. Correlation of the sensitivity to chemical structure of the organophosphates shows that introduction of one or two sulfur atoms into the pesticide molecules reduces the sensitivity of the instrument for it. CAM-1 is suited for monitoring of water for the presence of nonreversible cholinesterase inhibitors (i.e., nonreversible *in vitro*).

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FOREWORD

When energy and material resources are extracted, processed, converted, and used, the related pollutional impacts on our environment and even on our health often require that new and increasingly more efficient pollution control methods be used. The Industrial Environmental Research Laboratory - Cincinnati (IERL-Ci) assists in developing and demonstrating new and improved methodologies that will meet these needs both efficiently and economically.

This report describes the performance of the Cholinesterase Antagonist Monitor (CAM-1) for the detection of toxic and subtoxic levels of organophosphate and carbamate pesticides in water supplies on a real time basis. CAM-1 is intended to be used in the laboratory environment for monitoring raw water supplies entering a water treatment facility and checking effluents from pesticide manufacturing plants or related applications. It has the capability to warn of pesticide spills in time to permit corrective action to be taken. Information on this subject beyond that supplied here may be obtained from the Oil and Hazardous Materials Spills Branch (IERL), Edison, New Jersey 08817.

David G. Stephan
Director
Industrial Environmental Research Laboratory
Cincinnati

ABSTRACT

The Cholinesterase Antagonist Monitor (CAM-1) uses a pad of immobilized cholinesterase for the collection and detection of pesticides in water. A 3-min detection cycle provides an electrical readout proportional to the activity of the enzyme before and after its exposure to the pesticide sample. CAM-1 has been operated with water containing a variety of pollutants including organophosphates, carbamates, chlorinated hydrocarbons, and various other economic poisons; its sensitivity to these materials has been measured. With few exceptions, only the organophosphates and carbamates, which are nonreversible inactivators of cholinesterase, are detectable with CAM-1. One of these exceptions is zinc at 10 ppm, which inactivates cholinesterase and behaves in CAM-1 like the organophosphates. However, the zinc interference can be removed or taken into account. Another compound detectable under certain conditions is the reversible cholinesterase antagonist, tributylamine hydrochloride; it is detectable for only one or possibly two detection cycles when a sudden increase in the concentration of the reversible inhibitor occurs. The nonreversible enzyme inhibitors, on the other hand, produce repeated voltage increases until the enzyme in CAM-1 is completely inactivated. CAM-1 is especially suitable for the detection of nonreversible inhibitors.

Correlation of the sensitivity of CAM-1 with the chemical structures of a group of organophosphate pesticides has shown that CAM-1 is generally more sensitive for the phosphate (-O-P=O) compounds than for the phosphorothioates (-O-P=S) or the phosphorodithioate (-S-P=S) compounds even though the animal toxicities of these different types of compounds may be very close. A technique for the conversion of the -O-P=S and -S-P=S moieties into the corresponding -O-P=O form is proposed as a method to increase the sensitivity of CAM-1 to these particular types of compounds but this has not yet been studied. A portable device using the CAM-1 principle could be fabricated and used to locate spills, follow the spill plume, and to test treated water for its freedom of insecticides.

Operation of CAM-1 in salt water (3% NaCl) changes the voltage baseline (registered on the digital voltmeter and also recorded) but it does not change the sensitivity of CAM-1 for compounds like DDVP (dimethyl-dichlorovinyl-phosphate); thus CAM-1 is suitable for responding to cholinesterase inhibitors in either sea or brackish waters. CAM-1 has much promise for assessing the quality of water supplies and of plant effluents that may have been contaminated by spills or even chronic discharges of cholinesterase inhibitors, but obviously, prior to putting CAM-1 into regular service, more extensive testing is advisable under the conditions of intended usage.

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LIST OF ABBREVIATIONS

BuSChI	-- Butyrylthiocholine iodide
CAM-1	-- Acronym for Cholinesterase Antagonist Monitor
ChE	-- Horse serum cholinesterase
DDVP	-- O,O-Dimethyl 2,2-dichlorovinyl phosphate
HSChI	-- Thiocholine iodide
HEPES	-- N-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
THAM	-- Acronym for tris(hydroxymethyl)amino methane
Tris	-- THAM (see above)

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The work upon which this publication is based was performed pursuant to Contracts Nos. 68-01-0038 and 68-03-0299 with the Environmental Protection Agency and describes the work on Task I for the latter contract. Task II on this contract, "Construction of a Portable Enzyme Detection Apparatus," is in progress; Task III, "Alternative Enzyme Systems for Use in CAM-1" is authorized and is expected to be funded.

The authors wish to thank Mr. Lorren Kurtz, Mrs. Margo Rogers, and Miss Sandra Puent of Midwest Research Institute for their technical assistance. Also we wish to thank Dr. Thomas Hoover of EPA's Southeast Water Quality Laboratory, Athens, Georgia, and Dr. John E. Brugger of EPA's Industrial Environmental Research Laboratory - Cincinnati, Edison, New Jersey, for their technical assistance and encouragement. The generosity of the pesticide manufacturers in supplying samples for this study is appreciated.

SECTION I

INTRODUCTION

At the 1972 National Conference on Control of Hazardous Material Spills, MRI reported the development of a rapid detection system for organophosphates in water.[1] A subsequent report, essentially on the material contained in this document, was presented at the 1974 Conference.[2] The apparatus using this system (Figure 1) was identified as the Cholinesterase Antagonist Monitor (CAM-1) since it uses cholinesterase for collection, concentration, and detection of cholinesterase inhibitors present in water. At that time, this use of immobilized cholinesterase for automatic detection of organophosphates and carbamates in water was new. The final report of MRI's initial contract with EPA[3] contained the data in Reference 1 plus some additional details of construction of CAM-1 and its performance; the abstract from this document is reproduced as Appendix A of this report.

The objective of Task I was to determine the sensitivity of the CAM-1 instrument for specific pesticides including not only the organophosphates and carbamates but also the chlorinated hydrocarbons and various potential interfering substances. The studies were also intended to answer questions about the relative response of CAM-1 to reversible inhibitors vs nonreversible inhibitors. In addition, this contract provided the experience with an immobilized enzyme detection system necessary to prove its reliability and suitability for use in both laboratory and field applications. As shown in this report, CAM-1 does have application for the rapid detection of toxic or subtoxic levels of certain pesticides in water.

Work on Task II is in progress; no work has yet been done on Task III on this contract since it has not been funded. Task II is concerned with the fabrication of a portable model of CAM-1 and Task III is concerned with the use of a CAM-1 type apparatus with another enzyme system.

The gel-entrapped cholinesterase product used in the CAM-1 instrument for the detection of cholinesterase antagonists has already been described in the literature.[4] This product was prepared by coprecipitation of cholinesterase with aluminum hydroxide gel followed by entrapment in starch gel on the surface of open-pore polyurethane foam. The resulting sheets of treated foam were cut into pads which were 1/4 in. thick and 3/8 in. diameter so that they would fit into the CAM-1 electrochemical cell. These pads retain a major portion of their

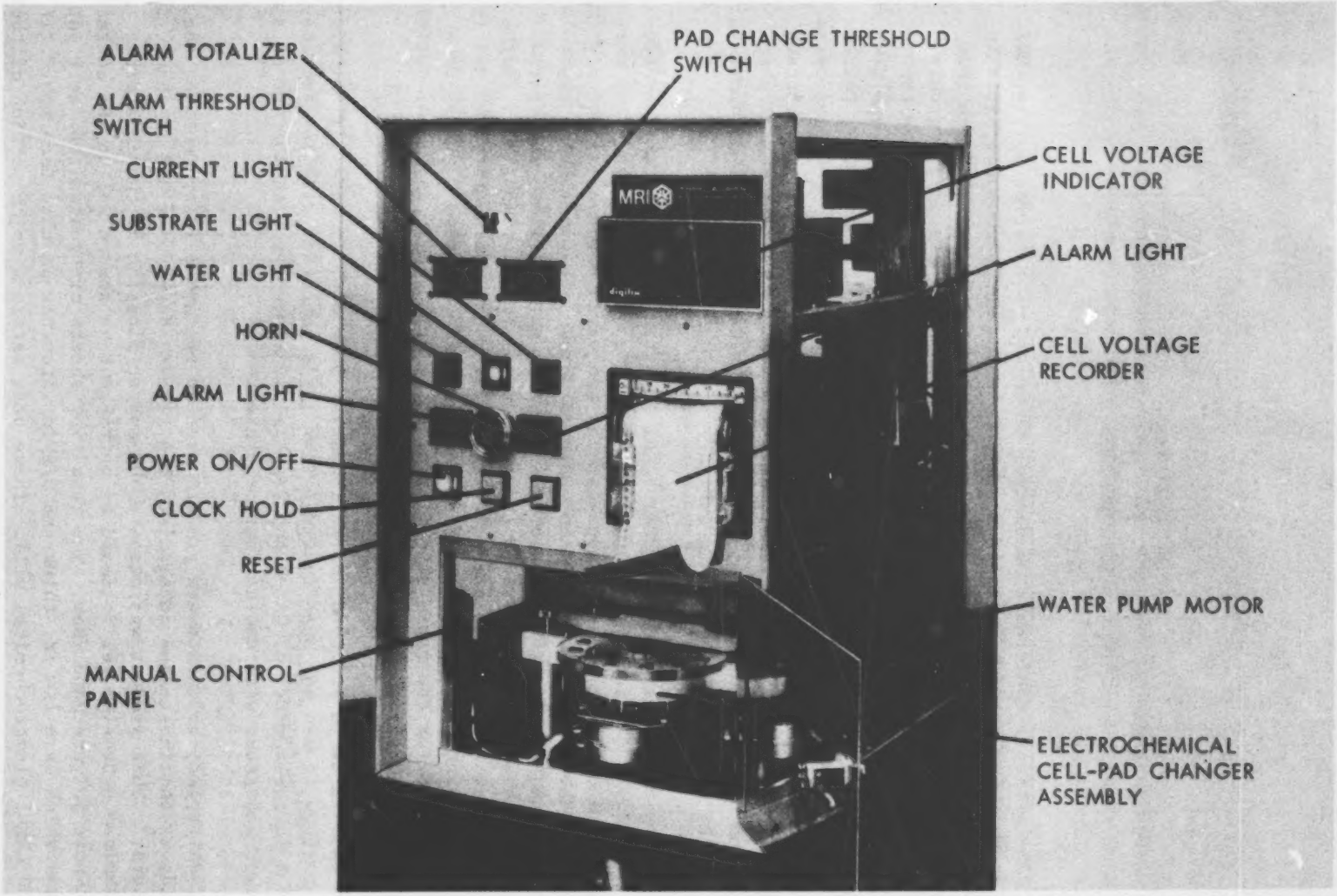


Figure 1. The Cholinesterase Antagonist Monitor, CAM-1, with cover removed.

activity after having been washed with water at 1,000 ml/min for 24 hr provided that the water temperature is 25°C or below and provided enzyme inhibitors are absent. Pads may also be used at higher water temperatures but their useful life is thereby reduced. A detailed procedure for the preparation of these enzyme pads is given elsewhere in this report.

Very briefly, the operating principle for CAM-1 is as follows: an electrochemical process is used for the automatic determination of the activity of the enzyme pad once during each 3-min cycle. As may be seen from the cross section of the electrochemical enzyme cell shown in Figure 2, the porous enzyme pad is located betwen two porous platinum electrodes. First the water sample is pumped through the enzyme pad at about 1,000 ml/min for 2 min during which time a portion of the enzyme inhibitor in the water combines with the active sites on the enzyme to reduce its enzyme activity. At the end of the 2-min water sampling period, the water is turned off and air is blown through the enzyme pad to displace the residual water. Next, a solution of substrate consisting of butyryl thiocholine iodide in Tris buffer is pumped through the enzyme pad at the rate of 1 ml/min for a period of 1 min; during the last two-thirds of this substrate pumping cycle a constant current of 2 μA is applied to the platinum electrodes so that the lower electrode is positive (anode) and the upper electrode is negative (cathode). In the absence of enzyme inhibitors, the cholinesterase (ChE) hydrolyzes the substrate to give thiocholine iodide, which possesses a thiol group that gives characteristic low voltages in the electrochemical cell.

$$C_3H_7COSCH_2CH_2\overset{+}{N}(CH_3)_3\ I^- \xrightarrow{ChE} C_3H_7COOH + HSCH_2CH_2\overset{+}{N}(CH_3)_3\ I^-$$

$$\downarrow \text{electrochemical oxidation}$$

$$(CH_3)_3\overset{+}{N}\text{-}CH_2CH_2\text{-}S\text{-}S\text{-}CH_2CH_2\overset{+}{N}(CH_3)_3\ 2I^-$$

On the other hand, in the case of total enzyme inhibition, substrate is not hydrolyzed; there is no thiol formation and the cell voltage rises about 200 mV (from about 200 mV to 400 mV). Thus, a low cell voltage is indicative of the absence of enzyme inhibitors, while an increase in cell voltage means that all or part of the enzyme activity has been removed by an inhibitor from the water sampled. Per cycle, increasing cell voltage means increasing inhibition.

The operation of CAM-1 is further illustrated by considering an example in which water containing 0.2 ppm of DDVP (dimethyl-dichlorovinyl-phosphate) is detected. The voltage tracing shown in Figure 3 was generated by applying a constant current to the enzyme pad once each cycle. The difference in voltage peak heights from cycle to cycle is used to trigger an alarm upon reaction of

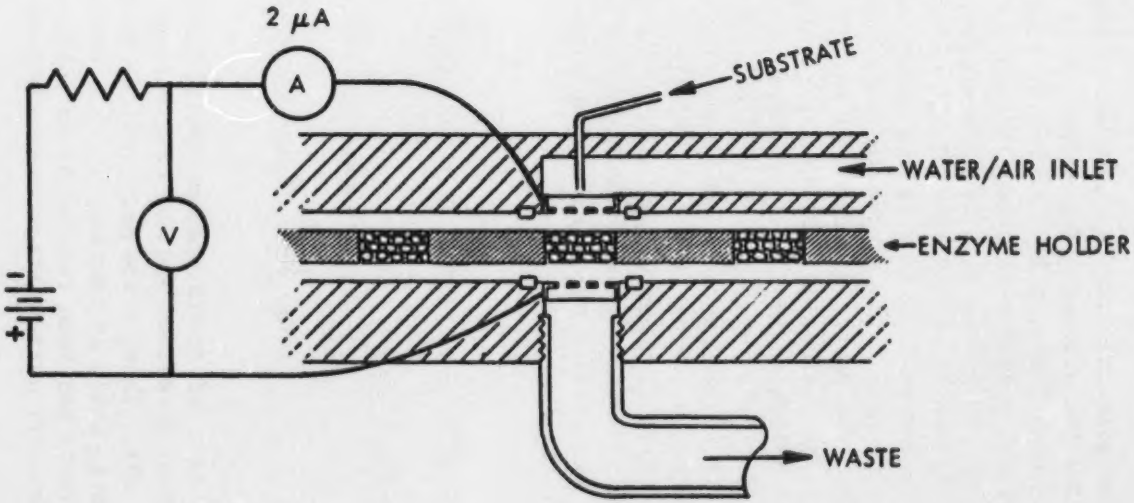


Figure 2. Cross section of electrochemical enzyme cell showing connections for the constant current supply and the electrometer.

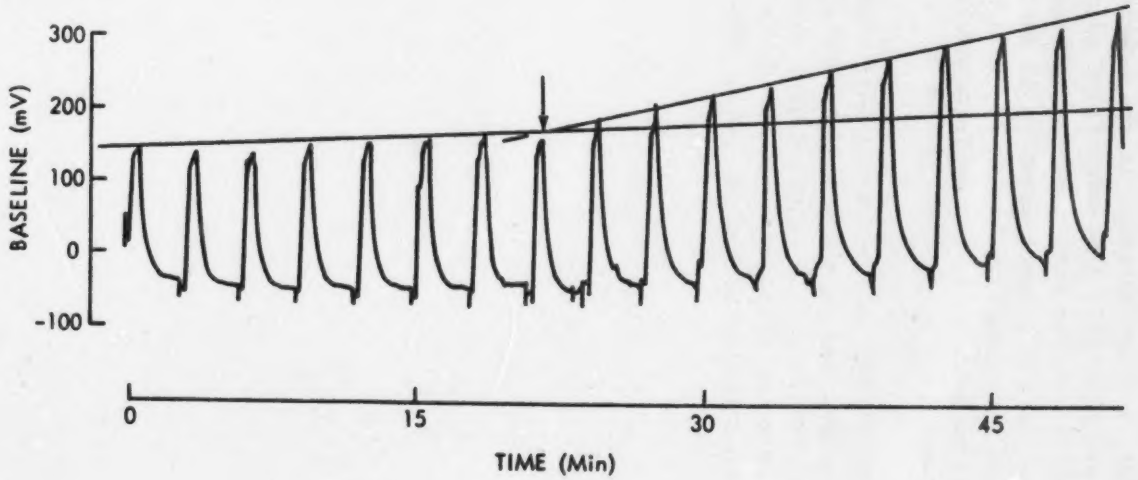


Figure 3. Response of the electrochemical cell operating on the 3-min cycle to water containing 0.2 ppm DDVP.

a cholinesterase inhibitor such as DDVP. (Note: Only the peaks are of interest. The decrease to the "resting" value of ca. -50 mV is not of importance in this discussion.) As shown in Figure 3, during the first 24 min, there is a very slow steady voltage rise from cycle to cycle, indicating the gradual deterioration of the immobilized enzyme pad. The alarm level is set so that these changes are too small to trigger an alarm. However, when 0.2 ppm of DDVP is added to tap water, a sharp increase in the height of the voltage peaks occurs. When the alarm threshold (or sensitivity adjustment) is set at 10 mV, then an alarm will be sounded each time the voltage increases by 10 mV or more between cycles. In the present example, 10 individual alarms from the same enzyme pad resulted from sampling the DDVP. If a higher concentration of DDVP--perhaps 2 ppm--had been used, the cycle-to-cycle voltage increases would have been much greater.

Enzyme pads cannot be used indefinitely. After a time, when there is insufficient enzyme activity on the enzyme pad to allow a 50 mV voltage rise when inhibitors are sampled, the used enzyme pad is rejected as a safety factor and a new pad is automatically inserted into the system by means of an enzyme-pad-changing mechanism built into the electrochemical cell. The system holds a total of 11 pads.

SECTION I

CONCLUSIONS

1. CAM-1 is essentially specific for organophosphate and carbamate insecticides and is able to detect spills of these hazardous materials at subtoxic or toxic levels within 3 min. CAM-1 can also be used to determine insecticide levels in continuous discharges such as manufacturing plant effluents.

2. Compounds with high affinity for cholinesterase are detectable at low concentrations, whereas compounds with lesser affinities are detectable at higher concentrations.

3. The sensitivity of CAM-1 to organic phosphates ($-O-\overset{|}{P}=O$) is often greater than to phosphorothioates ($-O-\overset{|}{P}=S$) and phosphorodithioates ($-S-\overset{|}{P}=S$).

4. CAM-1 at ambient temperature (20-25°C) responds to organic phosphate and carbamate pesticides in water at temperatures ranging from 5° to 35°C. At elevated temperatures, the CAM-1 cell voltages--a measure of enzyme activity--observed on the digital voltmeter are lower due to increased conductivity of warm water. DDVP (0,0-dimethyl 2,2-dichlorovinyl phosphate, acute oral LD_{50} to rats 56-80 mg/kg) can be detected at 1.0 ppm at 5°C and at 0.2 ppm at 25°C. Sensitivities for other pesticides were shown to exhibit similar changes with temperature.

5. Mixtures of two different pesticides each at one-half of its detectable concentration became detectable indicating the additivity of the response.

6. Operation of CAM-1 on raw Missouri River water, as it was entering the Kansas City, Missouri Municipal Water Plant, showed that there were no detectable quantities of organophosphates or carbamates in the water supply during a day of monitoring. Also, no interferences from species in the raw water to the electrochemical cell were noted.

7. The aluminum hydroxide/starch gel-entrapped cholinsterase on open-pore polyurethane foam products (referred to in this report as "enzyme pads") were made by different laboratory technicians working only with written instructions. The resulting pads functioned satisfactorily in CAM-1. In the present experiments, these pads kept CAM-1 operational for periods ranging from a few min to 24 hr (tests were limited to 24 hr), depending

upon the water temperature and the level of cholinesterase inhibitors present. In earlier tests, single enzyme pads lasted as long as 56 hr when used with tap water.

8. The butyrylthiocholine iodide used as the substrate was dissolved in Tris buffer, pH 7.4. When compounded with commercial Tris buffer from different suppliers, the substrate solution had only a useful life ranging from 6 to 12 hr. This useful life could be extended to 96 hr by the simple expedient of treating the Tris buffer with activated charcoal within a day or two prior to the preparation of the substrate solution.

9. A variety of possible interfering substances was tested to see whether they behaved like the organophosphate and carbamate cholinesterase inhibitors. The substances tested included other insecticides, herbicides, molluscicides, nematocides, and fungicides. None of these affected the CAM-1 baseline voltage sufficiently to produce an alarm.

10. Studies of the effect of inorganic salts were also conducted. In the case of zinc salts, there was nonreversible inhibition of cholinesterase and an alarm signaled at 10 ppm and greater concentrations. Although the presence of 3% salt (NaCl) lowered the cell voltages (because conductivity of the cell increased while the cell current was maintained at a constant value, namely, 2 μA) it did not change the sensitivity of the instrument to DDVP. This indicates that CAM-1 could be used to assess the level of organophosphate insecticides in brackish waters.

11. The present experiments with CAM-1 have demonstrated the feasibility of using immobilized cholinesterase to detect toxic levels of pesticides in water and suggest the need for a more portable, battery operated system for use at spills.

12. During this study CAM-1 instruments were operated for ~ 2,500 hr. There were no breakdowns or significant maintenance problems. These units even performed satisfactorily after shipment by air freight for four 1,000-mile trips.

Note: In the text--to conserve space and avoid repetition--organophosphate and carbamate pesticides (insecticides) are frequently referred to collectively as "organophosphates". Further, unless the text or context indicates otherwise, the specific term "organophosphates" includes organic phosphates ($-O-\overset{\uparrow}{P}=O$), organic phosphorothioates ($-O-\overset{\uparrow}{P}=S$), and organic phosphorodithioates ($-S-\overset{\uparrow}{P}=S$).

SECTION III

RECOMMENDATIONS

On the basis of data reported in this document, CAM-1 has been found suitable for assessing the quality of water to determine whether the water has been accidentally contaminated by organophosphate or carbamate insecticides. Use of CAM-1 is therefore recommended for checking water suspected of having been contaminated by spills of insecticides, as well as by the effluent from insecticide manufacturing operations, and by agricultural run-off, chronic discharges, etc. It has been shown that the sensitivity of CAM-1 varies with the individual organophosphate or carbamate; however, in each case the pesticides have been detected in water at both subtoxic and toxic levels so that water users may be protected from receiving an acute lethal dose of these pesticides.

Because field use of CAM-1 has been limited, it is recommended that CAM-1 be subjected to field testing under the conditions of anticipated use prior to putting it into regular service at a particular location.

In its present configuration, CAM-1 is not readily usable where electrical service is absent. MRI has recommended that a battery-operated model of CAM-1 be fabricated and field tested. This lightweight, portable device would be specifically designed to give rapid, in-field indications of cholinesterase inhibitors in water, to trace the contaminated plume of spilled organophosphate as it moved downstream, and to ascertain the level of organophosphates in the effluent from processing equipment that has been employed to strip the spilled pollutant from water.

Since horse serum cholinesterase has a lower affinity for those organophosphate pesticides which possess the phosphorothioate (-S-$\overset{|}{P}$=S) structures than for -O-$\overset{|}{P}$=O structures, it is recommended that further studies be conducted to optimize the sensitivity of CAM-1 to these -S-$\overset{|}{P}$=S materials. Several approaches to this problem which should be investigated include the following: (1) use of an oxidant in the pesticide solution, (2) pass the pesticide solution over an immobilized oxidase enzyme, (3) pass the pesticide solution over a solid oxidizer, or (4) find an enzyme which is more readily inhibited by these phosphorous-sulfur compounds and yet retain sensitivity to the -O-$\overset{|}{P}$=O compounds. Eel cholinesterase (E.C. No. 3.1.1.7) is known to be more readily inhibited by some organophosphates than horse serum cholinesterase; for this reason,

eel cholinesterase is judged to be one of those enzymes that should be investigated as a method for obtaining an increase in sensitivity of CAM-1.

Finally, since the basic CAM-1 concept may be useful in detecting other pollutants by using a different enzyme/substrate system, it is recommended that other specific enzyme systems can be studied. For example, it may be possible to utilize the pyruvate dehydrogenase/pyruvate/lipoic acid system in CAM-1 for the detection of pyruvate dehydrogenase inhibitors; in this system the -S-S- grouping of lipoic acid is reduced by pyruvate to -SH,-SH and this should be detectable electrochemically.

SECTION IV

ENZYME PAD PREPARATION PROCEDURE

Care must be taken in the preparation of the enzyme pad for use in CAM-1 since the sensitivity of CAM-1 and the repeatability of the tests are a function of the uniformity of the enzyme pads. Timing and manual dexterity are important procedural factors in the preparation of the pads. It is suggested that the individual selected to prepare the pads should practice the handling of the starch and its application to urethane foam as mentioned below, but with a water-soluble dye substituted for the enzyme. In this way, it will be possible to anticipate changes in starch viscosity and to check out the procedure for uniform distribution of the starch applied to the foam. After gelling and drying, the starch-coated foam should then be cut into pads, since starch pads are also needed for the determination of CAM-1's alarm potential at the beginning and ending of each days tests.

(The alarm potential is the voltage increase which occurs when all of the enzyme on the pad is inhibited or if the enzyme pad is replaced with a "starch pad" to which no enzyme has been applied. Daily checks of the alarm potential are suggested as a means to prove that the instrument is working.)

The following materials were used: open-pore polyurethane foam sheets, 44 to 45 pores per linear in. (ppi) x 1/4 in., Scott Industrial Foam, Scott Paper Company, Chester, Pennsylvania; partially hydrolyzed potato starch recommended for gel electrophoresis, Connaught Medical Research Laboratory, Toronto, Canada; Chlorhydrol® (aluminum chlorhydroxide complex, 50% w/w solution), Reheis Chemical Company, Chicago, Illinois; horse serum cholinesterase, Sigma Chemical Company, Type IV, approximately 15 μM units/mg; and Tris buffer, "THAM®," Fisher Scientific.

Step 1. A solution of Tris buffer, 0.08 M, was prepared by dissolution of 9.7 g of tris(hydroxymethyl)amino methane in 900 ml of water, adjustment of the pH to 7.4 with concentrated HCl and then adjustment of the volume to 1 liter.

Step 2. Forty milligrams of horse serum cholinesterase were dissolved in 6 ml of Tris buffer. To this solution was added with mechanical stirring 4 ml of a dilute solution of aluminum chlorhydroxide complex (0.5 g of Chlorhydrol® in 4 ml of water). At this point

the aluminum hydroxide gel precipitates and adsorbs the enzyme from the solution (check pH and adjust to 7.4 to ensure that the precipitation is complete). This suspension is set aside at ambient temperature until needed in Step 3.

Step 3. Two grams of potato starch were suspended in 10 ml of cool Tris buffer and added to 30 ml of boiling Tris buffer and heated until the suspension cleared. Care was taken to avoid the formation of scum or lumps (start Step 3 over if lumps are obtained) and the starch slurry was stirred with a magnetic stirrer while it cooled slowly to 45°C. At this point the aluminum hydroxide gel-entrapped cholinesterase (Step 2) was added all at once and quickly mixed. (Note: Step 4 and its three replications must be done quickly before the starch gels.)

Step 4. A 10-ml portion of the warm starch gel slurry from Step 3 was deposited on a pre-cut sheet of open-pore urethane foam (4 x 6 x 1/4 in. sheet) lying on a warmed glass or plastic surface (usually over a pan of warm water). The starch-enzyme material was now distributed throughout the sheet as uniformly as possible with a plastic rolling pin filled with warm water; the sheet was rolled in all directions and turned over several times. In this same manner, three other 10-ml portions of the starch-enzyme product were distributed over three additional urethane foam sheets.

Step 5. The coated sheets were placed on edge in a wooden rack (made with dowel rods), dried at least an hour at room temperature and finally dried overnight in an oven at 37°C.

Step 6. The resulting sheets were examined carefully to ensure that all areas of all pads were evenly coated. Poorly-coated areas were trimmed away. The sheets were handled carefully to avoid breakage of the starch film on the dried sheets. The enzyme pads were next cut into 3/8-in. pads with a stainless steel cutter (Figure 4) mounted in an electric drill press operated at about 600 rpm. The procedure yielded approximately 270 enzyme pads, which possessed an average activity of 0.045 μmoles/min/pad (analysis based on the rate of hydrolysis of butyrylthiocholine iodide and was measured by a modification of the Ellman Procedure).[4]

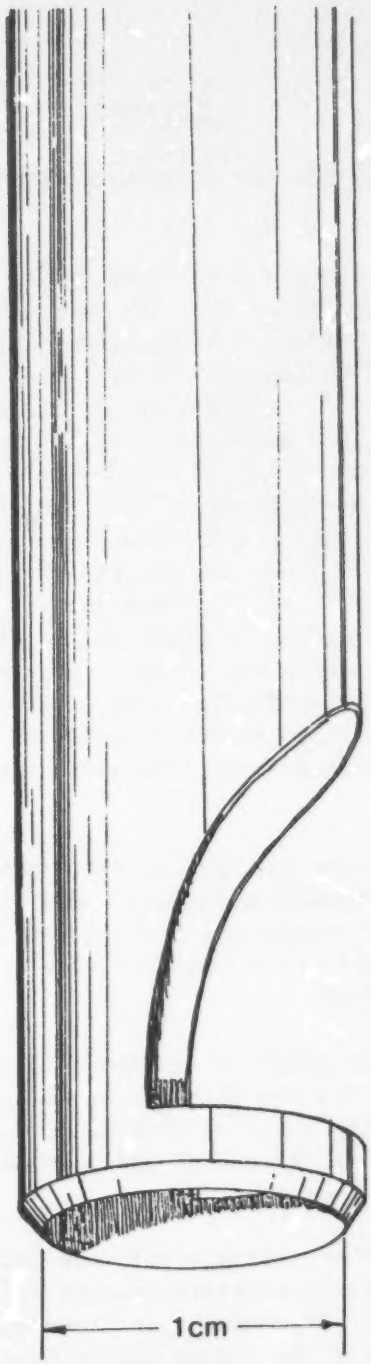


Figure 4. Enzyme Pad Cutter

SECTION V

BUFFER AND SUBSTRATE STUDIES

During the initial laboratory studies with CAM-1, it was observed that there was a deterioration of performance of the instrument when it was operated for 6 to 10 hr at a time. However, the performance appeared perfectly normal on subsequent days when the instrument was recharged with fresh substrate solution and a new enzyme pad. The observed deterioration of performance took the form of decreased starch pad and alarm potentials at the end of 8 hr of operation. At the start of one run, the initial voltage with a starch pad was 400 mV, the enzyme pad potential was 120 mV, and the alarm potential was 280 mV. After 8 hr of detector operation, the enzyme pad potential was 220 mV, the starch pad potential was 220 mV, and the alarm potential was 0 mV--obviously a dangerous situation since the sampling of an enzyme inhibitor at this time would give no voltage increase and no alarm signal. The problem was shown <u>not</u> to be related to the enzyme pad since insertion of a new pad did not restore the alarm potential. However, replacement of the substrate solution with freshly-prepared substrate solution restored the alarm potential to useful levels. For this reason, laboratory investigations of the substrate were undertaken.

One proposed explanation for the loss of alarm potential was that the substrate solution had undergone spontaneous hydrolysis and that the thiocholine iodide liberated by this process was keeping the starch pad voltage low. Two experiments were conducted to see whether the useful life of the substrate solution could be prolonged.

In one approach, a gentle stream of air was bubbled through the substrate solution for 8 hr during its use with CAM-1; this aeration of the solution was expected to oxidize the thiol as fast as it was formed by spontaneous hydrolysis and to keep both the starch pad voltage and the alarm potentials high. Aeration had no effect.

In the second approach, the substrate solution was cooled in ice with the expectation that the spontaneous hydrolysis of the thioester would be retarded at the lower temperature. Improvement in alarm potential during longer runs was noted; but, still, both the starch pad voltage and the alarm potentials decreased during the longer runs. (Note that some decrease in alarm potential due to the loss of enzyme activity of the enzyme pad is normal but does not lower the starch pad voltage or interfere with the operation of CAM-1 as now programmed.)

The third approach was based on the premise that the poor stability of the substrate solution arose from the presence of an impurity in the substrate, butyrylthiocholine iodide (BuSChI). A new sample of BuSChI was obtained from a second supplier and tested in CAM-1; this substrate solution also deteriorated in 6 to 8 hr. One commercial lot of BuSChI was recrystallized from isopropanol, washed with isopropanol, and dried in a vacuum; the white, odorless, crystalline product melted at 174°-176°C and was believed to be very pure based upon previous studies at MRI with this product, which included examination by thin-layer chromatography. The recrystallized BuSChI performed as well as, but no better than, the unrecrystallized BuSChI from which it had been prepared.

The Tris buffer was next evaluated as a possible source of the problem; three alternative buffers were investigated as replacements for the Tris buffer. The buffers investigated were 0.1 M phosphate, Veronal® (sodium barbital), and N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Calbiochem). These buffers were adjusted to pH 7.4 and then used for dissolving the BuSChI at a concentration of 2.5×10^{-4} M. Substitution of each of these substrate solutions for the corresponding substrate solution made with 0.08 M Tris buffer, pH 7.4, produced an operating detector system but resulted in the introduction of other problems, particularly an increased variability of the cycle-to-cycle pad voltages. For example, with the phosphate buffer and an enzyme pad, the cell voltage started at 104 mV and increased 72 mV during the first hour. With the HEPES buffer the enzyme pad voltage increased 72 mV in 45 min (with Tris buffer the voltage usually rises < 20 mV/hr). With the Veronal buffer, the buffer crystallized from the solution and plugged the substrate lines. These buffers offered no improvement over the Tris buffer.

It was learned that substrate (i.e., BuSChI) dissolved in Tris buffer which has been freshly-treated with activated petroleum-base charcoal gave satisfactory CAM-1 performance for at least 79 hr.

Several possibilities for the troublesome instability of the substrate solution were considered: (1) there was microbial growth in the buffer, (2) the dry buffer contained an impurity which caused the trouble, and (3) there was a slow chemical reaction occurring in the buffer solution when it was stored for a long period in air and exposed to light. The first explanation appeared to be incorrect since signs of microbial growth were not observed. The second explanation appeared to be wrong, since the initial treatment of the buffer with charcoal should have removed the impurity. The possibility of a slow (1 month or more) chemical reaction of the Tris buffer with air and light seems to fit the present observations. Additional studies are needed to obtain a more definite chemical explanation for the substrate instability observed in the initial experiments.

Data showing the reproducibility of CAM-1 performance from day to day and also the solution to the substrate instability problem are given in Table 1. The starch pad voltage, the enzyme voltage, and the alarm potentials both at the start and at the conclusion of the test runs are given for each day. The footnotes to the table give explanations for the meaning of the potentials measured. At the start of each run the alarm potentials were all over 250 mV, which is considered quite satisfactory. Comparison of the final starch pad voltages reveals that there were two runs in which there was a significant drop in potential after 8 to 8-1/2 hr of CAM-1 operation. In the first case, there was a drop in starch pad voltage from 410 to 218 mV; in this test, the charcoal treatment of the old Tris buffer solution within a few days of the run had been omitted. In the second case, the starch pad voltage fell from 435 to 344 mV; in this case freshly-made Tris buffer was used but the charcoal treatment was omitted. These experiments demonstrate that with some lots of THAM buffer charcoal treatment prior to use in making the substrate solution is essential to good CAM-1 operation. Even though the chemistry of the changes occurring in the substrate-buffer solution is unknown, the changes may be delayed for a day or two by a simple charcoal treatment of the buffer prior to the addition of the substrate.

Petroleum-based pelletized charcoal (MCB Co.) was used for the purification of the buffer. In this process the weight of charcoal was 1 to 2 times the weight of the Tris dissolved in the water and the mixture was stirred from 2 to 8 hr before filtering.

Table 1. RECORD OF CAM-1 OPERATION SHOWING SUBSTRATE FAILURE ON 8-7-73 AND EFFECT OF BUFFER PURIFICATION[a]

Test Date	Start			Final			Duration of Test, (hr)	Buffer			Enzyme Pad
	Starch Pad, mV[b]	Enzyme Pad, mV[c]	Alarm Potential, mV[d]	Starch Pad, mV[e]	Enzyme Pad, mV	Alarm Potential, mV		Age of Solution	Charcoal Treatment	Interval: Charcoal to Use	
8-3-73	404	112	289	408	229	179	7 1/4	old[f]	yes	0	11-29-72
8-6-73	378	64	314	390	180	210	8 3/4	old	yes	0	7-18-73
8-7-73	410	130	280	218[g]	123	95	8 1/2	old	no	---	7-18-73
8-8-73	404	98	306	409	175	234	7 1/2	old	yes	0	7-18-73
8-9-73	411	128	283	417	204	213	9	old	yes	0	7-18-73
8-10-73	418	134	284	419	190	220	8 1/2	old	yes	0	7-18-73
8-13-73	411	92	319	424	211	233	8 3/4	old	yes	4 days, 5°C	7-18-73
8-14-73	427	121	306	454	156	298	10 1/2	old	yes	1 day, 25°C + 4 days, 5°C	7-18-73
8-15-73	439	143	296	438	389	49	10 1/2	old	yes	4 days, 5°C + 2 days, 25°C	7-18-73
8-20-73	397	102	295	412	165	247	8 1/2	old	yes	0	7-18-73
8-21-73	415	123	292	415	214	201	9 3/4	old	yes	1 day, 25°C	7-18-73

(Continued)

Table 1 (continued). RECORD OF CAM-1 OPERATION SHOWING SUBSTRATE FAILURE ON 8-7-73 AND EFFECT OF BUFFER PURIFICATION[a]

Test Date	Start			Final			Duration of Test, (hr)	Buffer			Enzyme Pad
	Starch Pad, mV[b]	Enzyme Pad, mV[c]	Alarm Potential, mV[d]	Starch Pad, mV[e]	Enzyme Pad, mV	Alarm Potential, mV		Age of Solution	Charcoal Treatment	Interval: Charcoal to Use	
8-22-73	431	105	326	423	127	296	7 3/4	old	yes	0	7-18-73
8-23-73	418	147	271	430	177	253	8	old	yes	0	7-18-73
8-24-73	423	117	306	424	256	168	8	old	yes	0	7-18-73
8-27-73	392	77	315	405	187	218	8	old	yes	5 days, 25°C	7-18-73
8-28-73	410	110	300	420	270	150	8	old	yes	0	7-18-73
8-30-73	429	137	292	435	262	229	8	new	yes	0	7-18-73
8-31-73	435	155	280	3448	215	129	8	new	no	---	7-18-73

[a] CAM-1 was operated at room temperature with 2.5×10^{-4} M BuSChI (Pierce Chemical Company) in 0.08M Tris buffer, pH 7.4. All the Tris buffer was Fisher Scientific Certified Reagent of the same lot number.

[b] Cell voltage obtained with a starch (no enzyme) pad in the cell.

[c] Cell voltage obtained with an enzyme pad.

[d] Alarm potential is the voltage increase expected when the enzyme in a pad is completely inhibited.

[e] The final starch pad potential should not drop during a test run.

[f] Solutions prepared more than a month were called "old". All had been charcoal treated when first made.

[g] These starch pad voltages are abnormally low.

SECTION VI

CAM-1 OPERATION AND PERFORMANCE DATA

The length of time that CAM-1 may be operated without servicing depends upon several variables. The automatic pad changer has slots for only 11 enzyme pads, but this situation is not expected to restrict the period of operation between servicing periods, except in unusual circumstances where multiple spills occur in a short period of time or when a high level of cholinesterase inhibitor persists for a long period of time. The pads themselves possess sufficient enzyme so that each will operate the detector for 24 to 48 hr so long as nonreversible enzyme inhibitors are absent. (Enzyme is slowly washed out, limiting the useful performance of a pad.) Under ideal conditions (i.e., when no inhibitors are present), 11 enzyme pads should last 11 to 22 days. In the presence of highly-polluted water, the pads will not last so long. The substrate used in this instrument is butyrylthiocholine iodide (BuSChI) at a concentration of 2.5×10^{-4} M in 0.08 M Tris buffer, pH 7.4. Unfortunately the substrate undergoes changes in solution and these spontaneous changes probably will limit the period of service-free operation. When the substrate is prepared carefully in Tris buffer which has been freshly treated with activated charcoal, and when the temperature is kept at 25°C or below, the substrate will provide good alarm potentials for 48 to 96 hr. For longer operation it might be better to refrigerate the substrate solution or to devise an automatic solution maker that will add solid substrate to fresh buffer at intervals of a day or less. In this way, operation for a week between service periods could be assured. In the laboratory, it was convenient to prepare new substrate solution and add new enzyme pads daily.

The cost of the materials used for operation of the instrument is of interest. The horse serum cholinesterase used for our laboratory studies cost $67.50 for 1,000 µM units; however, since each enzyme pad contains only 0.01 to 0.05 units/pad, it is clear that the enzyme for a single pad costs less than $0.01. When labor for pad fabrication and analysis is included with the cost of all materials, the enzyme pads might cost from $0.03 to $0.10 each depending on the quantities prepared in a single batch. The ingredients for the substrate solution are expected to cost no more than $0.20/day and most of this cost is for the buffer.

Preliminary field testing has been conducted with CAM-1 primarily to determine the background response that might be encountered with raw water supplies. In one case, water from a freshwater lake was monitored from a floating dock extending 30 ft from the shore; there was no sign of enzyme inhibition and one enzyme pad provided satisfactory baseline voltages for more than 8 hr. In another test, CAM-1 was taken to the Kansas City Municipal Water Works where it was used to monitor the incoming raw water for an 8-hr day. In this case, there was no evidence of enzyme inhibitors entering the water supply and no false alarms when the threshold sensitivity was set at 10 mV. The next step in field testing will be to evaluate the performance of the system with effluents from pesticide manufacturing operations.

Studies on the repeatability of CAM-1's response to constant levels of pesticides in water have been made. These studies were primarily aimed at finding out whether the sensitivity of the instrument changed with time.

In some of the initial studies with Baygon® it was observed that 16 ppm was detectable with fresh solutions whereas solutions five times as concentrated were barely detectable after 24 hr. The loss in sensitivity was attributed to the partial hydrolysis of the Baygon®.

In a 79-hr test of substrate stability, CAM-1 was exposed repeatedly to a solution of 2.5 ppm of Diazinon®. Table 2 shows the changes in the electrochemical cell potentials at the end of each 3-min detection cycle.

From the data in Table 2, it is clear that there was either a slight decrease in sensitivity as the substrate solution became old or that there was some hydrolysis of the stock solution used in making the 19-liter batch of Diazinon® test solution. The unusually large voltage changes occurring after the 5th cycle in Test 3 and after the second cycle in Test 7 suggest that mixing of the solution was inadequate; in subsequent experiments, the pesticide solutions were mechanically stirred and there was greater uniformity of cycle-to-cycle voltage changes. However, examination of the voltage changes for each test makes it clear that enzyme inhibition occurred in all of the tests.

During the course of the study the CAM-1 instruments were operated for approximately 2,500 hr. Very few mechanical or electrical problems were encountered. Occasionally it was necessary to "free" (manually turn) the water pump before startup when the unit has been turned off for several days. The rubber shaft seals on the water pumps had to be replaced once. In one of the CAM-1 units, there was a failure of a digital in-line packaged integrated circuit (DIP IC) compatible relay and a new relay had only to be plugged in to restore the normal detection cycle. On one occasion a fuse in the water pump circuit blew; replacement with a "slow-blow" fuse put the system back in operation and no additional troubles were observed. Changes in the wiring diagrams or components were not required.

Table 2. RESPONSE OF CAM-1 ON REPEATED CHALLENGE WITH WATER CONTAINING 2.5 PPM DIAZINON®

Test No.	Voltage Response for Each Cycle Following Exposure to Diazinon (mV)						Detectable at 2.5 ppm[b]	Test Temperature (°C)
	1[a]	2	3	4	5	6		
1	7	32	30	23	21	21	Yes	23
2	-6	25	12	16	17	16	Yes	23
3	-4	10	12	13	43	--	Yes	23
4	8	22	18	14	31	43	Yes	21
5	-2	12	7	8	10	--	Borderline	21
6	6	37	43	21	11	3	Yes	21
7	21	51	14	28	10	--	Yes	21
8	5	46	45	29	26	--	Yes	21
9	10	23	19	25	35	26	Yes	21
10	-12	12	21	12	16	11	Yes	21
11	1	30	24	15	29	--	Yes	21
12	-1	8	18	13	12	21	Yes	21
13	-16	17	26	12	12	22	Yes	21
14	-12	15	8	12	5	12	Borderline	21
15	7	5	17	16	18	37	Yes	21
16	-3	12	24	21	6	27	Yes	21
17	-9	19	-1	10	10	--	Borderline	21
18	-2	8	10	11	-1	14	Borderline	21

[a] The voltage change during the first cycle was not reliable due to the lack of temperature compensation in CAM-1 and the fact that the containers of water with and without the Diazinon® were not always at exactly the same temperature.

[b] Assumes that the alarm threshold would be set at 10 mV/cycle.

SECTION VII

EFFECT OF TEMPERATURE ON SENSITIVITY OF CAM-1

Since CAM-1 is expected to operate with water ranging in temperatures from 5° to 35°C, it is important to know how temperature affects the electrode potentials and also the sensitivity of CAM-1 to various pesticides. It is well known that water with dissolved salts is a better electrical conductor when warm than when cold due to the greater mobility of ions in the warm solution. In the same manner, the movement of ions through the enzyme pad to the electrodes in contact with it is faster with warm water and slower with cold water. As a result of this conductivity effect and of the constant-current system design, higher cell voltages are obtained when the cell is cold and lower cell voltages when the cell is warm. Within the normal operating temperatures, these relationships are of no concern unless there is a sudden temperature change of the water sampled; a decrease of 3°C in the water temperature within one detection cycle (3 min) could result in a 10-mV increase in baseline voltage and a false alarm, provided the alarm threshold was set at 10 mV. Because of the high heat capacity of water, it is not expected that rapid changes of water temperature will be a problem. Temperature compensation of the baseline voltage is possible but it is not included in the present CAM-1.

When the CAM-1 instrument is operated at 25°C with water at 5°C, it is likely that the temperature of the electrochemical cell during the last minute of the detection cycle is significantly higher than 5° since the substrate solution is also at 25°C.

Quite a different problem is the effect of temperature on the reaction of the enzyme with the substrate and with the inhibitors. A thorough study of this problem has not been made since classical biochemical investigations have already established the mathematical relationships of temperature to enzyme reactivity. However, limited studies have been made on the effect of temperature on the total CAM-1 system--that is, the sum total of all temperature effects occurring in the total detection system. The kinds of temperature effects to be seen are best illustrated by considering one specific example, i.e., the effect of temperature on the sensitivity of CAM-1 to DDVP as shown in Table 3.

Table 3. EFFECT OF WATER TEMPERATURE ON THE SENSITIVITY OF CAM 1 TO DDVP

Test No.	Level of DDVP (ppm)[a]	Voltage Increase for Each Cycle, mV					Detectable at Test Level	Temperature (°C)[c]
		1	2	3	4	5		
1	1	21	33	26	31		Yes	6
2	1	12	30	18	20		Yes	6
3	1	60	76	55			Yes	14
4	1	98	99	47			Yes	14
5	0.2	2	13	10	15	6	Yes	14
6	1	46	93	74			Yes	25
7	1	64	86				Yes	25
8	1	48	78	71			Yes	25
9	0.3	26	28	27	35	31	Yes	25
10	0.2	5	11	15	14	15	Yes	25
11	0.1	9	5	6	5	8	Borderline[b]	25
12	1	88	59	41	15	10	Yes	35
13	0.2	17	16	11	8	8	Yes	35
14	0.1	10	11	8	9	10	Borderline	35

[a] The Vapona® used in this experiment contained 93% DDVP and 7% related phosphates.

[b] Borderline means that cumulative enzyme inhibition is observable and that the voltage increase between cycles averages more than 5 mV.

[c] Water temperature studies were done in laboratory at 25°C.

Comparison of the cycle-to-cycle voltage increases produced by DDVP at 1 ppm and at temperatures of 6°, 14°, 25°, and 35°C shows sharply increased voltage changes at the higher temperature; this is attibuted to an increase in the rate of enzyme inhibition at the higher temperatures. In Test No. 12, it will be seen that large voltage changes were observed for the first three detection cycles but that the magnitude of the changes decreased for subsequent cycles, even though DDVP at 1 ppm was still present for all cycles. The explanation for this anomaly is that nearly all of the enzyme was inhibited during the first three cycles and the cell was approaching the maximum voltage which could be obtained when no enzyme is present. Under normal CAM-1 operation, the pad change threshold would have been set so that a new pad would have been inserted automatically after the third detection cycle.

Based on studies with DDVP and also other suitable insecticides, it is concluded that CAM-1 is about two times more sensitive at 25°C than it is at 5°C. However, even at the lower temperature, CAM-1 retains sufficient activity to detect both toxic and subtoxic levels of most insecticides with anticholinesterase activity.

SECTION VIII

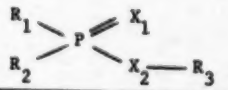
RESPONSE OF CAM-1 TO ORGANOPHOSPHATES AND CARBAMATES

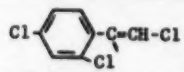
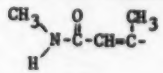
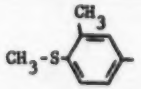
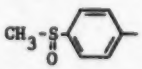
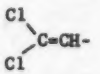
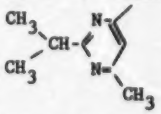
As mentioned earlier, the principal objective of the present study was to determine the potential usefulness of CAM-1 for which the sensitive element is immobilized cholinesterase. A previously uninvestigated area was whether the sensitivity of CAM-1 for a specific insecticide is a function of the affinity of cholinesterase for that insecticide. In other words, is it true that compounds with a high affinity for cholinesterase are detectable at low concentrations, whereas, compounds with lesser affinities for cholinesterase are detectable only at higher concentrations. CAM-1 has now been operated with solutions or suspensions of about 40 economic poisons to determine CAM-1's ability to detect the poisons.

Table 4 presents the CAM-1 response data collected with a group of 22 organophosphates. The data are arranged so that it is convenient to make correlations between the structural characteristics of the pesticides and the minimum detectable levels. From the table it is apparent that the greatest sensitivity was obtained with paraoxon (detectable at 0.1 ppm) and the least sensitivity to Di-Syston® (detectable at 65 ppm). In general, CAM-1 exhibited greater sensitivity for the phosphates and phosphonates (-O-$\overset{|}{P}$O-compounds) than for the phosphorothioates and dithioates (-O$\overset{|}{P}$S- and -S-$\overset{|}{P}$S-compounds). The relative insolubility of these compounds makes it difficult to know in every case that the enzyme inhibition was due to dissolved, rather than dispersed, pesticide; for this reason, caution is urged in making comparisons of relative concentrations producing enzyme inhibition.

For these response studies, commercial grades of the named compounds were dissolved or suspended in city tap water at several concentrations and pumped through CAM-1 at known temperatures. For some of the experimental evaluations, the very insoluble materials were dissolved in alcohol and added slowly to a stirred container of water. The resulting clear solutions, opalescent solutions, or milky suspensions were then pumped through CAM-1 at about 1,000 ml/min. The lowest concentration of organophosphate producing a cell voltage increase of at least 10 mV/cycle was considered to be detectable at this level and at the temperature of the test. After each test, the pesticide solutions were hydrolyzed with caustic prior to their disposal. Tests with carbamates, chlorinated hydrocarbons, and other pollutants were conducted in much the same manner, although caustic could not be used to destroy the dilute chlorinated hydrocarbon wastes.

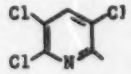
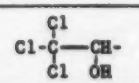
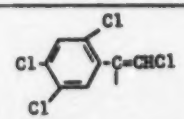
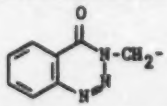
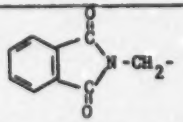
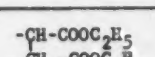
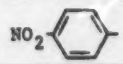
Table 4. CORRELATION OF ORGANOPHOSPHATE INSECTICIDE STRUCTURES AND DETECTABILITY WITH CAM-1



Common Name	R_1	R_2	X_1	X_2	R_3	Detectable Level, ppm	T., °C	Acute oral LD_{50} rats,[b] (mg/kg)
Aktone®	C_2H_5O-	C_2H_5O-	S	-O-		12	12	146
Azodrin®	CH_3O-	CH_3O-	O	-O-		20	7	23
Baytex®	CH_3O-	CH_3O-	S	-O-		22.5	20	male 190 female 310
Dasanit	C_2H_5O-	C_2H_5O-	S	-O-		3	20	male 10.5 female 3.2
DDVP	CH_3O-	CH_3O-	O	-O-		0.2	25	56-80
Diazinon	C_2H_5O-	C_2H_5O-	S	-O-		1.2	23	150-220
Di-Syston®	C_2H_5O-	C_2H_5O-	S	-S-	C_2H_5-S-CH_2CH_2-	65	25	10

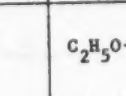
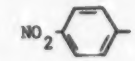
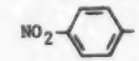
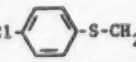
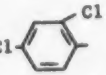
(Continued)

Table 4 (continued). CORRELATION OF ORGANOPHOSPHATE INSECTICIDE STRUCTURES AND DETECTABILITY WITH CAM-1

Common Name	R_1	R_2	X_1	X_2	R_3	Detectable Level, ppm	T., °C	Acute oral toxicity rate, (mg/kg)
Dursban®	C_2H_5O-	C_2H_5O	S	-O-		4.5	21	male 160 female 135
Dyfonate®	C_2H_5O-	C_2H_5-	S	-S-		15	10	16.5
Dylox®	CH_3O	CH_3O	O	-O-		0.8	20	450-500
Gardona®	CH_3O-	CH_3O-	O	-O-		0.75	13	4,000-5,000
Guthion®	CH_3O-	CH_3O-	S	-S-		12.5	13	male 80 female 16
Imidan®	CH_3O-	CH_3O-	S	-S-		22	25	216
Malathion	CH_3O-	CH_3O-	S	-S-		17-70[a]	25	4,000
Methyl Parathion	CH_3O-	CH_3O-	S	-O-		26	9	9-25

(Continued)

Table 4 (continued). CORRELATION OF ORGANOPHOSPHATE INSECTICIDE STRUCTURES AND DETECTABILITY WITH CAM-1

Common Name	R_1	R_2	X_1	X_2	R_3	Detectable Level, ppm	T., °C	Acute oral toxicity rats, (mg/kg)
Monitor®	CH_3O-	CH_3-S-	S	-NH_2	-----	20	20	20
Nemacur®	C_2H_5O-		O	-NH-		0.36	10	8
Paraoxon	C_2H_5O-	C_2H_5O-	O	-O-		0.1	7	3.5
Parathion	C_2H_5O-	C_2H_5O-	S	-O-		5-10[a]	10	5.0
Systox®	C_2H_5O-	C_2H_5O-	S	-O- and -S-	C_2H_5-S-CH_2CH_2-	1.4	20	7.5 and 1.7
Trithion®	C_2H_5O-	C_2H_5O-	S	-S-		10	13	32
VC-13®	C_2H_5O-	C_2H_5O-	S	-O-		10	8	270

[a] Responses from different lots of pesticides varied.

[b] Most of the toxicity data was from the Pesticide Index.[5]

Table 5 shows the sensitivity of CAM-1 to seven carbamate pesticides. Furadan® was detectable at 0.75 ppm and Temik® was detected at 0.5 ppm, which indicates very strong anti-cholinesterase activity for these carbamates--approximating the toxicity of the organophosphates with the P=O linkage. The other five carbamates tested in this study were detected at concentrations low enough to protect personnel using the water supplies from acute toxic doses of these carbamates.

The previous tests were conducted using water solutions of pure pesticides. The addition effects of two or more pesticides were demonstrated by challenging CAM-1 with a solution containing one-half the detectable concentration of DDVP and one-half the detectable concentration of paraoxon. The response of CAM-1 to the combination was equivalent to challenges by detectable concentrations of either. Experiments using other pairs of pesticides confirmed the additivity rule, as did one experiment in which a combination of three pesticides was used: parathion, paraoxon, and DDVP.

Table 5. REPRESENTATIVE CARBAMATES DETECTED BY CAM-1

Trade Name	Chemical Name	Concentration Detected, ppm	Acute oral LD_{50} rats, (mg/kg)
Baygon®	o-Isoproxyphenyl N-methylcarbamate	20	male 104 female 95
Dimetilan	2-Dimethylcarbamoyl 3-methylpyrazo-5-yl dimethylcarbamate	10	47-64
Furadan®	2,3-Dihydro-2,2-dimethylbenzofuran-7-yl N-methylcarbamate	0.75	5
Mesurol®	4-(Methylthio)-3,5-xylyl N-methyl-carbamate	12	100
Sevin®	1-Naphthyl N-methylcarbamate	20	400
Temik®	2-Methyl-2-(methylthio)-propionaldehyde O-(methylcarbamoyl)oxime	0.5	1
Metacil®	4-Dimethylamino-3-tolyl N-methylcarbamate	7	30

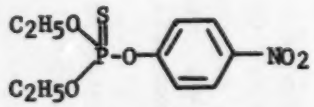
SECTION IX

CORRELATION OF PESTICIDE DETECTABILITY WITH PESTICIDE TOXICITY

For most organophosphate and carbamate insecticides, both their toxicities in warm blooded animals and their detectability on CAM-1 are directly related to the affinity of the insecticide for the enzyme. This relationship exists because the mechanism of toxicity and the mechanism of the detection process are both dependent upon the blockage of active sites on the cholinesterase molecule. For example, a compound such as paraoxon is quite toxic in animals and is detectable in low concentrations in water with the CAM-1 system.

The mechanism by which these insecticides are able to inhibit cholinesterase is well known and is reported in literature reviews of cholinesterase prepared by Froede and Wilson[8] and by Metcalf.[6] In man and also in CAM-1, the organophosphate insecticides phosphorylate one of these active sites (i.e. the esteratic site) on cholinesterase to inhibit its enzymatic activity. In man, the cholinesterase is essential for the hydrolysis of the acetyl choline which hydrolysis results in the relaxation of muscles after contraction. Inhibition of the action of this enzyme in man, therefore, produces toxic symptoms which include contraction of the pupils, nausea, muscular rigidity, convulsions, etc.

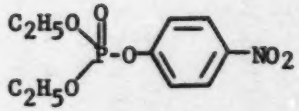
For some organophosphate and carbamate pesticides, however, the correlation between animal toxicities and detectabilities by CAM-1 is poor. This is especially true for those compounds that are classed as phosphoro-thioates, phosphoro-dithioates and thiocarbamates. These compounds are less strongly attracted to cholinesterase and are therefore less readily detectable by the CAM-1 system. On the other hand, compounds in this group retain their toxicity to warm blooded animals since their metabolism in the liver (oxidation) produces the more toxic oxygen analogues. As shown in Figure 5, organophosphates such as parathion and malathion are very toxic because they are oxidized to the oxygen analogues <u>in vivo</u>. As shown in Table 4, many of the commercial organophosphates do have the phosphoro-thioate and dithioate structures (i.e., $-O-\overset{|}{P}=S$ and $-S-\overset{|}{P}=S$). Chemically their reduced affinity for cholinesterase is due to the fact that compounds with the P=S linkage are less electrophilic than compounds with the P=O linkage.[6]



PARATHION

IN VIVO

Oxidase and Coenzyme



PARAOXON

Oral Toxicity (Rats, LD_{50})	Female:	1.75-5.0 mg/kg	3.5 mg/kg
	Male:	5-30 mg/kg	3.5 mg/kg
Minimum Detected with CAM-1:		10 ppm *	0.1 ppm

* No conversion of parathion to paraoxon in CAM-1.

Figure 5. Comparison of toxicity and detectability of parathion and paraoxon.

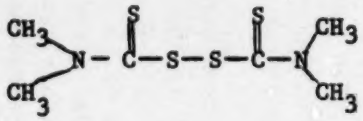
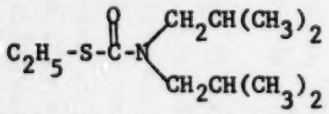
It should be noted that a common impurity in the commerical phosphoro-thioate and dithioate insecticide products is the oxygen analogue of the insecticide; because of the greater affinity of the impurity for the enzyme, significant differences in the detectability of the commercial pesticides can exist. Parathion (P=S type) from two suppliers has been detected at 5 to 10 ppm, whereas the corresponding oxidized material (i.e., paraoxon, P=O type) is readily detected at 0.1 ppm. On the other hand, the toxicities of these two compounds in rats are essentially the same. The sensitivity of CAM-1 to the P=S type of organophosphates is less than that to the P=O type of compounds. Nevertheless, CAM-1 can detect the P=S type of insecticides at concentrations that will prevent water supply users from receiving acute oral doses of these pesticides. Promising ideas for increasing the sensitivity of CAM-1 to the P=S compounds by an order of magnitude have been generated but have not yet been investigated sufficiently to report on at this time. Basically, the idea is to pre-oxidize the P=S type compounds to the P=O type.

SECTION X

RESPONSE OF CAM-1 TO REVERSIBLE CHOLINESTERASE INHIBITORS

Several reversible inhibitors of cholinesterase have been encountered in this study of the response of CAM-1 to a variety of potential water pollutants. Reversible inhibitors have the ability to inhibit cholinesterase when these inhibitors are present in moderate concentrations; however, they neither bind tightly nor do they phosphorylate the enzyme; therefore, they are not concentrated in the enzyme pad to the same extent as the nonreversible inhibitors. Table 6 provides some response data on three such reversible cholinesterase inhibitors. Thiban® 75 (tetramethylthiuram disulfide) is an animal repellent and fungicide, and, judging from its chemical structure, it would not be expected to form covalent bonds with the active sites of the enzyme. However, at 30 ppm this material produced a voltage increase of 45 mV during the first cycle, with very small increases during subsequent cycles. Changing of the CAM-1 inlet hose to freshwater resulted in the washing out of the inhibitor, the reactivation of the enzyme, and the return of the electrochemical voltage to its pre-exposure value. A similar result was obtained with tributyl amine (TBA) hydrochloride, which should add reversibly to the "anionic site" (i.e., cation attracting site) of cholinesterase (i.e., the quaternary nitrogen of acetylcholine chloride). As shown, 100 ppm of tributyl amine caused a 38-mV increase on the first cycle and only small increases on subsequent cycles. On introducing freshwater into CAM-1, the TBA was washed out of the enzyme pad slowly and five wash cycles were required to obtain the original pre-exposure voltage. A third example of a reversible inhibitor is Sutan®, which is S-ethyl diisobutylthiocarbamate. The voltage increase in the first detection cycle is a function of the concentration; little or no voltage increase is noted in subsequent detection cycles. From these data (Table 6), it is apparent that detection of reversible inhibitors is likely to occur only when there is a sudden change from no inhibitors to fairly high levels of the reversible inhibitors. In such cases, only one cycle will show a large voltage increase and subsequent cycles will show either small responses or none. A voltage drop with no alarm signal will be noted when the reversible inhibitor is removed by washing the freshwater. In passing, it should be emphasized that low levels of reversible inhibitors do <u>not</u> prevent the detection of the nonreversible cholinesterase inhibitors.

Table 6. RESPONSE OF CAM-1 TO REVERSIBLE CHOLINESTERASE INHIBITORS

	Conc., ppm	CAM-1 Voltage Changes Per Cycle, mV
 Thiban® 75 (fungicide)	30	45, 9, 9, 4, 9, 6
$(C_4H_9)_3N \cdot HCl$		
Tributylamine HCl (industrial chemical)	100	38, 7, -1, 9, 2, 2
	42	20, -4, 0, -2, -3,
Sutan® (herbicide)	100	30, 10, -7, 9, -8,

SECTION XI

STUDIES OF CAM-1 WITH OTHER ECONOMIC POISONS AND DISSOLVED SALTS

The various non-phosphate, non-carbamate pesticides investigated included herbicides, defoliants, insecticides, nematocides, fungicides, rodenticides, repellents, and others. Some specific compounds tested with CAM-1 included aldrin, chlordane, dieldrin, lindane, chlorophenoxyacetate salts, piperonyl butoxide with pyrethrins, Daconil (tetrachloroisophthalonitrile), Thiophanate [diethyl 4,4'-O-phenylenebis (3-thioallophonate)], Fumasol-C® [sodium salt of 3(α-acetonylfurfuryl)-4 hydroxycoumarin], Phaltan® (N-trichloro-methyl-thiophthalamide), etc. None of these economic poisons had any effect on the cell voltage in CAM-1 and thus were not detectable.

Only very preliminary studies of the effect of dissolved inorganic materials in water on the performance of CAM-1 have been made and these are shown in Table 7. Surprisingly, zinc sulfate at 10 ppm caused repeated alarms until the enzyme pad was exhausted. Reports of zinc as an inhibitor of cholinesterase were not found; however, there are some enzymes, such as carbonic anhydrase which require zinc and other enzyme systems which require either calcium or magnesium ions in which inhibition by zinc occurs because of a displacement of the calcium or magnesium ions. Since cholinesterase does require calcium ions, its replacement by zinc may be the mechanism of cholinesterase inhibition but this has not been investigated. Detection of potassium dichromate by CAM-1, if it had occurred, would not have been surprising since dichromate is known to denature proteins by a tanning reaction and since it could also produce a rise in the electrochemical cell voltage simply by oxidation of the thiol liberated from the substrate of the enzyme. No alarms were produced by this compound; neither was the enzyme denatured nor was the substrate oxidized (most of the dichromate was apparently removed from the electrochemical cell prior to the addition of the substrate). Experiments with sodium arsenate showed that it could not be detected at 10 ppm.

Mercuric chloride at 10 ppm produced alarms for the first two out of six detection cycles. The effect on the system was similar to that observed for the reversible enzyme inhibitors. Low levels of mercuric ions in water would not produce an alarm in CAM-1 although a situation in which there were rapidly fluctuating levels of mercury might cause an occasional alarm. Field tests on polluted streams will be required to determine whether reversible inhibitors in the water cause significant problems, and these are recommended.

Table 7. EFFECT OF INORGANIC SALTS ON PERFORMANCE OF CAM-1

Compounds Tested	Level Tested (based on active ingredient, ppm	Voltage Response for Each Cycle Following Exposure, mV						Detectable at Test Level[c] (Temp, °C)
		1	2	3	4	5	6	
Calcium chloride	10	-8	-6	-2	0	-6	-	No (20°C)
Zinc sulfate	10	35	23	15	20	7	-	Yes (9°)
Potassium dichromate	10	-3	-7	4	-3	0	-	No (8°)
Sodium arsenate	10	-8	-2	-2	-15	-2	-	No (5°)
Mercuric chloride	10	22	14	3	2	4	0	Reversible (5°)
Sodium chloride	30,000 (3%)	-40	-1	0	0	0	0	No (12°)

Sodium chloride has but little effect on cholinesterase activity of the enzyme pads. However, switching of the CAM-1 rapidly from freshwater to 3% salt water causes a 40-mV drop in cell voltage. After this voltage drop, the voltage remained essentially unchanged for the next five cycles. The observed voltage change is attributed to the presence of more ions in the enzyme pad after the thorough washing in 3% salt water. Because of the porous nature of the starch gel, this extra ionic material is not all removed in 1 min during the enzyme pad testing portion of the cycle. So long as the salinity of the water being tested changes slowly, no problems in monitoring fresh, brackish, or sea water for the presence of enzyme inhibitors are anticipated. Tests with 0.1 ppm DDVP in 3% salt water at 9°C showed that it was detectable by CAM-1; this is the same sensitivity we observed in freshwater at 6°C.

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APPENDIX A

A RAPID DETECTION SYSTEM FOR ORGANOPHOSPHATES AND CARBAMATE INSECTICIDES[3/] IN WATER

ABSTRACT

An apparatus for the detection and monitoring of water supplies for hazardous spills of organophosphate and carbamate insecticides has now been designed and fabricated. The new unit is called the Cholinesterase Antagonist Monitor, CAM-1, because it produces an alarm in 3 min when toxic or subtoxic levels of cholinesterase antagonists are present in water. Response of this apparatus to subtoxic levels of Azodrin®, Sevin®, dimetilan, malathion, parathion, and DDVP has already been demonstrated. CAM-1 uses immobilized cholinesterase for the collection of cholinesterase inhibitors from the water supplies. The activity of the immobilized cholinesterase is determined automatically in an electrochemical cell by passing a substrate solution over the enzyme at regular time periods. A minicomputer is used to automate the detection process and to signal an alarm when there is a rapid loss of enzyme activity--a situation which occurs in the presence of organophosphate and carbamate insecticides in the water sampled.

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APPENDIX B

MECHANISM OF ELECTROCHEMICAL DETECTION PROCESS IN CAM-1

1. Oxidation Potential Theory. This theory proposes that cholinesterase is able to convert butyrylthiocholine iodide (BuSChI) into butyric acid and thiocholine iodide (HSChI) and since the thiol is easily oxidized a low voltage is measured by the constant current electrochemical system. In the absence of enzyme there is no thiol present and the voltage rises--usually about 250 mV since the original substrate BuSChI is not readily oxidized. It has been proposed that the voltage setting reaction is the oxidation of I^- to I_2; this explanation has appeal but it does not explain (1) how this iodide-to-iodine oxidation potential can vary from 100 mV to 600 mV as the electrodes are conditioned; (2) no trace of iodine color has ever been detected on the starch covered enzyme pad and (3) why the voltage does not immediately fall to zero since traces of iodine completely depolarize the electrodes.

   Supporting this theory is the knowledge that HSChI is readily oxidizable to the disulfide and it can be found among the products coming through the electrochemical cell. If this theory is correct, the voltage change should be observable with a number of electrode pairs at equivalent solution concentrations. We have not found alternate electrode materials which work as well as platinum.

2. Anode Depolarization Theory. This theory suggests that the anode is coated with a layer of platinum oxides or sulfides and perhaps other materials which tend to reduce its electrical conductivity. Exposure of this coated anode to a solution containing a trace of thiol results in a depolarization or increase in conductivity of the anode coating. In favor of this theory is the finding that application of a direct current to two identical electrodes for a few minutes in the presence of hydrolyzed substrate (i.e., HSChI) results in the making of stable dissimilar electrodes which generate voltage like a battery when they are placed in an electrolyte. The electrodes are readily made alike or depolarized by treatment with a trace of free iodine or chlorine; such treatment in our electrochemical system drives the voltage to zero indicating excellent conductivity. The electrodes recover after the halogen is gone.

In beaker experiments a standard calomel electrode is used as a reference electrode while current is applied to two identical platinum electrodes in a solution; when the solution is changed, from HSChI to BuSChI, it is noted that nearly all of the voltage change occurs at the anode. This suggests that the conductivity of the anode surface is changing with the change of material in the beaker. Measurement of applied current in the electrochemical cell with an enzyme pad showed that it was sufficient to oxidize only about 5% of the HSChI produced by the enzyme pad. Presumably a close balance between coulombs of applied current and moles of HSChI would be required to obtain rapid response of the system to enzyme inhibitors.

The disulfide of thiocholine iodide found in the products coming from the cell could arise either from air or electrochemical oxidation of the HSChI. Hence no evidence is gained for either theory. With freshly-plated platinum electrodes, the voltages obtained with enzyme pads are often as low as 0 mV at first, and after the electrodes have been used for a while (e.g., a day or two), the enzyme pad voltage may be as high as 250 to 300 mV; at the same time the voltage change obtained on replacing an enzyme pad with a pad without enzyme is 200 mV or more whether the electrodes are new or conditioned. This suggests that the enzyme pad voltages obtained are not characteristic of the oxidation potential of thiocholine iodide since they range from 0 to 300 mV. In summary, the exact mechanism of the electrochemical reaction is unknown and both electrode polarization and thiocholine iodide oxidation may be occurring simultaneously in the electrochemical cell. The mechanisms enabling electrochemical estimation of enzyme pad activity are worthy of further investigation.

Further support to the anode depolarization theory is given by Kramer, et al.,[7] who reported constant current experiments in which depolarization of a platinum anode by thiocholine iodide resulted in increased conductivity of the anode. This electrochemical reaction forms the basis of their procedure for anlaysis of cholinesterase and thiocholine esters.

3. Measurement of Cell Voltages. During the last minute of the 3-min detection cycle, the relative activity of the enzyme pad is determined electrochemically. While the substrate solution is pumped continuously over the enzyme pad at ~ 1 ml/min, air at ~ 1 liter/min is also passed through the enzyme pad. During the final 40 sec of this substrate pumping cycle, a constant current of ~ 2 μA is applied to the platinum electrodes with the lower electrode being made positive. The cell voltage (as measured with a high-impedance voltmeter or electrometer) rises rapidly at first but then either approaches or in some cases passes a maximum voltage prior to the end of the cycle. Whether the voltage is sampled and shifted into the memory immediately before or after the peak voltage occurs makes

little difference in the performance of the CAM-1 provided the substrate flow is constant and provided that the constant current is applied for a constant period of time. Repeatability rather than attainment of a true equilibrium voltage was the basis for applying the constant current for 40 sec during each detection cycle.